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Expression, purification, and characterization of *Clostridium botulinum* type B light chain

Janice Gilsdorf, Nizamettin Gul, Leonard A. Smith *

Integrated Toxicology Division, U.S. Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Frederick, MD 21702-5011, USA

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Abstract

A full-length synthetic gene encoding the light chain of botulinum neurotoxin serotype B, approximately 50 kDa (BoNT/B LC), has been cloned into a bacterial expression vector pET24a+. BoNT/B LC was expressed in *Escherichia coli* BL21.DE3.pLysS and isolated from the soluble fraction. The resultant protein was purified to homogeneity by cation chromatography and was determined to be >98% pure as assessed by SDS–polyacrylamide gel stained with SilverXpress and analyzed by densitometry. Mass spectroscopic analysis indicated the protein to be 50.8 kDa, which equaled the theoretically expected mass. N-terminal sequencing of the purified protein showed the sequence corresponded to the known reported sequence. The recombinant BoNT/B light chain was found to be highly stable, catalytically active, and has been used to prepare antisera that neutralizes against BoNT/B challenge. Characterization of the protein including pH, temperature, and the stability of the protein in the presence or absence of zinc is described within. The influence of pH differences, buffer, and added zinc on secondary and tertiary structure of BoNT/B light chain was analyzed by circular dichroism and tryptophan fluorescence measurements. Optimal conditions for obtaining maximum metalloprotease activity and stabilizing the protein for long term storage were determined. We further analyzed the thermal denaturation of BoNT/B LC as a function of temperature to probe the pH and added zinc effects on light chain stability. The synthetic BoNT/B LC has been found to be highly active on its substrate (vesicle associated membrane protein-2) and, therefore, can serve as a useful reagent for BoNT/B research.
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Keywords: Botulinum neurotoxin; Protease; Light chain

Botulinum neurotoxin, the causative agent of botulism poses a significant bioweapon threat because of its extreme lethality. An outbreak of botulism caused by dispersion of toxin is a serious public health emergency that requires an immediate response by the administration of botulinum antitoxin and often mechanical ventilation. Botulism is a neuroparalytic disease caused by seven immunologically distinct neurotoxins (types A-G) produced by *Clostridium botulinum*, a gram-positive, rod shaped, motile, non-encapsulated, spore-forming anaerobic bacterium. Botulinum neurotoxin(s) (BoNT)[1] are expressed as single polypeptide chains with approximate molecular masses of 150 kDa. Most of the *C. botulinum* strains have endogenous proteases that nick the toxin at a protease-sensitive site, activating the toxin and generating a light chain and a heavy chain held together by a single disulfide bond. The light chain can be separated from the heavy chain by reduction followed by electrophoresis or chromatography. The smaller (50 kDa) N-terminal fragment is designated as the light chain (LC) while the C-terminal fragment (100 kDa)

* Corresponding author. Fax: +1 301 619 2348.
E-mail address: Leonard.Smith@AMEDD.Army.Mil (L.A. Smith).

[1] *Abbreviations used:* BoNT, botulinum neurotoxin; LC, light chain, HC heavy chain; ECL, electrochemiluminescence; IPTG, isopropyl-β-D-thiogalactopyranoside; TB terrific broth; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay, BCA, bicinchoninic acid; cIEF, capillary isoelectric focusing; CD, circular dichroism; PBS, phosphate-buffered saline.

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14. ABSTRACT
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is referred to as the heavy chain (HC) [1]. The zinc-endopeptidase catalytic domain of the toxin resides in the 50 kDa N-terminal portion of the active protein.

After binding to peripheral cholinergic nerve cells, the toxin is internalized into endosomes through receptor-mediated endocytosis [2,3]. The amino terminal half of the HC is believed to participate in the translocation mechanism of the LC across the endosomal membrane [4–6]. Upon internalization into vesicular compartments, the catalytic LC is translocated to the cytosol where the final event of intoxication involves the catalytic hydrolysis of key synaptic vesicle proteins [7–9] by the light chain [10,11]. The zinc-dependent endoproteolytic LC selectively inactivates three essential proteins referred to as SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptors) proteins which are involved in the docking and fusion of acetylcholine-containing vesicles to the plasma membrane [12,13]. Botulinum neurotoxins (BoNT) A-G each have the capacity to cleave a protein of the neuroexocytosis apparatus: SNAP-25 is cleaved by BoNT/A [14], BoNT/C, and BoNT/E [15,16]. Serotype C also cleaves syntaxin [17,18]. The LC of BoNT/B cleaves VAMP/synaptobrevin [11]; serotype D, G, and F are also specific for VAMP/synaptobrevin [19–21]. Inactivation of SNAP-25, syntaxin, or VAMP by BoNT leads to an inability of the nerve cells to release acetylcholine, resulting in neuromuscular paralysis [22]. The LC by itself is nontoxic and does not translocate though the cell membrane of cholinergic cells.

Our laboratory has produced BoNT/B LC as a reagent to be used in high-throughput assays to screen for potential LC antagonists, to further elucidate the toxin's mechanism of action, and to study the immunological response to the catalytic domain of the toxin. The LC was cloned into an *Escherichia coli* expression system, pET24a+, and the recombinant plasmid was transformed into BL21.DE3.pLysS cells. The LC was purified by successive cation-exchange chromatographic steps and characterized for purity, structural integrity, and enzymatic activity. Details of the production process and features of the recombinant BoNT/B LC are described within this study.

Materials and methods

Materials

All buffer reagents and components were from Sigma (St. Louis, MO) unless otherwise specified. Precast tricine gels, load buffer, running buffer, stains, molecular markers and oligonucleotides for the PCR reaction were obtained from Invitrogen (Carlsbad, CA). Plasmid pBlueScriptII was purchased from Stratagene (LaJolla, CA). Plasmid pET24a+ was purchased from Invitrogen (Carlsbad, CA). The *E. coli* strain DH5α used in the cloning procedure and *E. coli* strain BL21.DE3.pLysS used for protein expression were purchased from Novagen (Madison, WI). Electrochemiluminescence (ECL) reagents were purchased from Perkin Elmer Life Sciences (Boston, MA). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA) and used according to the manufacturer's directions. The chromatography columns and resins used for purification were from Applied Biosystems (Foster, CA) and Pharmacia (Uppsala, Sweden). Anti-botulinum serotype B-specific polyclonal equine antibodies were used to verify protein expression. The equine polyclonal antibodies were obtained from Per-Immune (Rockville, MD) for the Department of Defense. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Novagen (Madison, WI). The synthetic substrate peptide for type B LC contains residues 60-94 of human VAMP-2: acetyl-LSELDDRADALQAGASQFETSAA KLKRKYWWKNLK-carboxamide was custom synthesized by SynPep (Dublin, CA).

Construction of the synthetic gene BoNT/B LC

Oligonucleotides were designed using the published sequence for Okra *C. botulinum* structural gene encoding the type B neurotoxin and were used in a series of ligation/PCRs to generate a final 1323-base pair (bp) fragment that was cloned into pBlueScript II at *Xho*I–*Xba*I sites. This enabled the synthesis of single-stranded DNA that was used for DNA sequencing. Verification of the resultant clone was then confirmed by DNA sequencing. Plasmid DNA used for cloning and single-stranded sequencing was prepared by using a kit purchased from Qiagen (Valencia, CA). The sequence was optimized for GC content and codon usage. Approximately 21% of the published sequence was altered by codon optimization. Oligonucleotides were designed that contained the restriction sites for *Nde*I and *Bam*HI to facilitate the cloning process. The full-length gene was excised from vector pBlueScript II and subcloned into *Nde*I and *Bam*HI digested pET24a+ vector. The insert was ligated into pET24a+ so as to begin expression with the initial methionine of the LC. Sequencing of the complete clone was performed and a single mutation was noted. In vitro mutagenesis was performed to correct the misincorporation, the correction was verified by sequence analysis. The resulting construct was used to transform, by calcium phosphate precipitation, *E. coli* BL21.DE3.pLysS cells for protein expression. Clones were assayed by Western blot for their ability to express BoNT/B LC.

Expression and purification of the LC

The bacteria was cultured in 1-L flasks containing terrific broth (TB) supplemented with 60 μg/ml of kanamycin with vigorous shaking (220 rpm) at 37 °C until the cultures reached an OD_{600} of 0.6. The recombinant BoNT/B LC protein was induced by adding IPTG (final concentration 0.1 mM) for 18 h. Cells were harvested by centrifugation and the pellet was immediately used or

stored frozen at −20 °C. One gram of LC cell paste was resuspended in 20 ml of buffer (20 mM Tris, 2 mM EDTA, pH 5.0). The suspended cells were sonicated using an alternating cycle of sonication (30 s) and ice incubation (30 s) over a period of 12 min. To remove debris and insoluble material the supernatant was centrifuged 15 min, 4 °C, 15 K. Additional buffer, 20 mM Tris, 2 mM EDTA, pH 5.8, was added to a final volume of 40 ml. The supernatant was sterilized with 0.2 μm filters. Following the filtering process the soluble portion was further purified using cation exchange chromatography and a BioCAD Model 700E (Perceptive Biosystems, Farmingham, MA).

A Poros HS 20 column was equilibrated with buffer (20 mM Tris, 2 mM EDTA, pH 5.8) before loading the protein. Protein was eluted from the column with a linear gradient of 20 mM Tris, 2 mM EDTA, 1 M NaCl, pH 5.8, 0–100% over 30 min at a rate of 1 ml/min. Throughout the gradient 1 ml fractions were collected. The peak eluted over several fractions which were collected and pooled. The column was washed extensively and was equilibrated with buffer (20 mM Tris, 2 mM EDTA, pH 5.4). The pooled fractions of run 1 were equilibrated to the new buffer. The protein was loaded on the Poros HS 20 column to further purify. The pooled fractions of run 2 were collected. A Source 15 S column was equilibrated with 20 mM Tris, 2 mM EDTA, pH 5.4, and the pooled fractions from run 2 were loaded through the buffer port. A linear gradient of 20 mM Tris, 2 mM EDTA, 1 M NaCl, pH 5.4, 0–100% over 30 min at a rate of 1 ml/min was performed. The peak fractions of run 3 were collected, pooled, and assayed for homogeneity. The expressed and purified recombinant BoNT/B LC was stored at −70 °C in 20 mM Na acetate buffer with a pH of 5.8 containing 2 mM EDTA. Recovery of the BoNT/B LC was calculated to be 4 mg/g of cell paste.

Protein assays and SDS–PAGE

Total protein concentrations were determined by using bovine serum albumin as a protein standard and Pierce BCA (bicinchoninic acid) protein assay using the microscale protocol as the manufacturer directed. Electrophoresis was performed according to Laemmli [23] on a 10% acrylamide gel under reducing conditions with a Novex Mini-cell II apparatus (Novex, San Diego, CA); pre-stained SeeBlue markers were used to determine the size of the recombinant protein. Gels were stained and proteins were visualized using Coomassie brilliant blue R-250 and SilverXpress. Protein samples were further analyzed by Western blot analysis to confirm protein identity [24]. Proteins separated by SDS–polyacrylamide gels were transferred onto nitrocellulose followed by incubation with equine polyclonal antibody to BoNT/B toxin. Membranes were washed and then incubated with affinity purified goat anti-horse IgG (H + L) (Kirkeguaard & Perry Laboratories, Gaithersburg, MD) horseradish peroxidase antibody. Detection was accomplished by using an enhanced chemiluminescence system (ECL).

N-terminal sequence and mass spectroscopic analysis of rBoNT/B LC

Following SDS–PAGE electrophoresis of the purified LC, the resolved protein was transferred onto a PVDF membrane. The appropriate PVDF band was sequenced using Edman degradation in an Applied Biosystems Procise Sequencer in the 0- to 20-pmol range. Molecular mass was determined by Matrix-assisted laser desorption/ionization (MALDI)-TOF Analysis. Tryptically digested BoNT/B LC was co-crystallized with α-cyano-4-hydroxycinnamic acid (Agilent Technologies, Palo Alto, CA) and spotted directly on a stainless steel matrix-assisted laser desorption ionization (MALDI) plate. Mass spectra were acquired using an Applied Biosystems 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). For all mass spectra the laser frequency was 200 Hz. MALDI spectra were internally calibrated (<20 ppm) using trypsin autolysis products. Post-acquisition baseline correction and smoothing was carried out using software provided with the instrument. Spectra were submitted to Mascot (http://matrixscience.com) for peptide mass fingerprinting. Surface enhanced laser desorption/ionization (SELDI)-TOF analysis was also performed. Purified LC was spotted on a normal phase ProteinChip array and allowed to air dry. The ProteinChip spot was washed with an excess of water and sinapinic acid (Agilent Technologies, CA) was applied and allowed to dry. Mass spectra were acquired with a PBS-IIc SELDI-TOF mass spectrometer (Ciphergen Biosystems, Fremont, CA) using the ProteinChip software provided with the instrument with the following instrument settings: laser power 220, sensitivity 8, focus mass 50,000.

Isoelectric focusing

The isoelectric point of BoNT/B LC was determined using the imaged capillary isoelectric focusing (cIEF) method with the iCE280 system from Convergent Bioscience [25]. Focusing was performed using 100 mM H_3PO_4 as anolyte, 100 mM NaOH as catholyte, a focusing voltage of 3 kV and a focusing time of 5 min. Initial standardization of the instrument was performed using hemoglobin standard from Convergent Bioscience (Toronto, Canada). A working ampholyte solution was prepared by mixing 80 μl (100%) of broad range ampholyte pH 3–10 (Amersham Biosciences, Piscataway, NJ) to 920 μl of 0.5% methyl cellulose solution. The sample mix was prepared by mixing 176 μl of this working ampholyte solution, 2 μl of prediluted p*I* marker 7.0 and 2.0 μl of marker 8.6 (Bio-Rad, Hercules, CA). Twenty microliters of the protein sample was added to this mixture to make a total volume of 200 μl. Injection volume was 40 μl and the exposure time and current settings were 45 ms and 8.75 A, respectively.

Focusing time was done for 7 min. Four runs were made and the initial values for the p*I*, obtained in pixels, were calibrated into p*I* using known markers, i.e., 7.0 and 8.6.

UV–visible absorption, circular dichroism, and fluorescence measurements

Before each experiment, to determine protein concentration and assess its purity UV–visible absorption spectra were recorded at 20 °C with a Hewlett-Packard 8452 diode array spectrophotometer. LC concentration was determined using $A^{0.1\%}$ (1 cm light path) value of 1.0 at 278 nm [26]. Circular dichroism spectra were recorded at 20 °C and temperature-dependent unfolding of LC was followed by monitoring circular dichroism (CD) at 222 nm on a Jasco J-810 spectropolarimeter with quartz cuvettes of 1 mm path length. To increase signal-to-noise ratio an average of five scans were recorded at a scan speed 20 nm/min with a response time of 4 s. For all measurements a buffer blank was recorded and subtracted from sample recordings. A MW_r of 50975 for the 441-residue LC yielded the mean residue weight as 115.59. Tryptophan fluorescence emission spectra were recorded at 20 °C in a PTI QuantaMaster spectrofluorimeter, model RTC 2000 equipped with a Peltier controlled thermostat and Felix software package. Emission and excitation slit widths were set at 1 nm and excitation wavelength at 295 nm. An average of five scans was recorded for each spectrum. The solution properties of the peptide were investigated by CD [27–29].

Enzymatic activity assay of BoNT/B LC

Before each experiment, aliquots of the protein were passed through a PD-10 gel-filtration column equilibrated with 10 mM Na-phosphate, pH 6.5, to remove EDTA present in the storage buffer. The enzymatic activity assay was based on HPLC separation and measurement of the cleaved products from a 35-mer synthetic peptide corresponding to human VAMP-2 residues 60–94 (HV35) [27]. BoNT/B LC catalyzes the hydrolysis of this peptide between residues 17 (glutamine) and 18 (phenylalanine) corresponding to residues Q76 and F77 of human VAMP-2. Assay mixtures (30 μl) containing 0.18 mM substrate, 50 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) pH 7.2, 0.25 mM $ZnCl_2$, 5 mM DTT, and 1.0 μg light chain were incubated at 37 °C for 5 min. Assays were stopped by adding 90 μl of 0.7% trifluoroacetic acid. The amounts of uncleaved substrate and the products were measured after separation by reverse-phase HPLC. Solvent A consisted of 0.1% trifluoroacetic acid and solvent B consisted of 70% acetonitrile/0.1% trifluoroacetic acid. The flow rate was 1.0 ml/min at 25 °C and the gradient profile was as follows: 20% B (2.5 min); linear gradient to 80% B (21 min); 100% B (6 min). Kinetic parameters of the synthetic substrate were calculated from Lineweaver–Burke plot of initial rates of proteolysis by BoNT/B LC versus various peptide concentrations ranging from 0.016 to 0.1 mM.

Fragmentation of recombinant light chain

BoNT/B LC was passed through a PD-10 column to remove EDTA and collected in 50 mM Na-phosphate pH 6.5. The LC was mixed in the presence or absence of 0.5 mM $ZnCl_2$ and aliquots (30 μl) of the LC were distributed in screw-capped eppendorf tubes. The final concentration of the protein in each incubation tube was 0.35 mg/ml. The tubes were incubated at 4 and 23 °C. At specified time intervals 20 μl of 2× SDS-load buffer was added to a 30 μl aliquot for SDS–PAGE analysis.

Immunogenicity studies

Three groups of 20 mice were used for each protection assay. Survivor data is recorded as the number of survivors from the total number of animals tested. Animals used for the study were female Crl:CD-1 mice, 16–22 g, on receipt (Charles River, Wilmington, MA). Groups were inoculated three times at 0, 2, and 4 weeks, with 5 μg or 15 μg of immunogen (BoNT/B LC) per mouse. Injection volume was 100 μl per mouse. Intramuscular injection into the caudal thigh muscle mass with 0.2% Alhydrogel in 0.8% saline with 0.8% benzyl alcohol as a preservative was performed. Intra-ocular bleeds were performed on anesthetized mice to collect sera 2 days after the last injection. Mice were challenged 7 days after the final inoculation with 10^2 MLD_{50} or 10^3 MLD_{50} BoNT-B toxin from *C. botulinum* strain Okra (Metabiologics, Madison, WI) in gel-phosphate buffer (0.4% dibasic phosphate with 0.2% gelatin). Naïve mice were challenged with the same levels of toxin and used as control. Mice were observed daily and the number of mice that survived after 5 days was recorded. Research was conducted in compliance with the Animal Welfare Act and other federal statues and regulations relating to animals and experiments involving animals and adheres to principals stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where the research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Enzyme-linked immunosorbent assay (ELISA)

Immulon 2 plates (Dynatech, Chantilly, VA) were coated with botulinum neurotoxin type B (Metabiologics, Madison, WI) at 2 μg/ml, 100 μl/well, in phosphate-buffered saline (PBS) pH 7.4. The plates were incubated overnight in a humidity box at 4 °C. Diluted serum was added in duplicate to toxin-coated wells (100 μl/well). The secondary antibody was horseradish peroxidase conjugated goat anti-mouse IgG mAb and ABTS substrate was added as color developer (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The absorbance was measured with a microplate reader at 405 nm. Naïve mouse serum was added as a

negative control in each assay. The titer was defined as the reciprocal of the last dilution with an absorbance $\geqslant 0.2$ above background absorbance.

Results and discussion

Construction of the BoNT/B LC expression system

Previously, tetanus toxin fragment C had been expressed in *E. coli* at 3–4% cell protein. The sequence for *Clostridium tetani* was examined and it was found to contain rare *E. coli* codons encoding fragment C. When the coding sequence was replaced by sequence optimized for codon usage in *E. coli*, it had been shown that the expression of fragment C is increased approximately 11–14% [31]. Others have used a strong promoter of phage T_7 and physiological control to improve the level of expression of a synthetic BoNT/B LC [32]. In the present study we utilized both codon optimization, which was performed to limit reduced protein expression associated with rare codons and high AT base composition [33,34], and a strong T_7 promoter. The use of a strong promoter and optimized synthetic BoNT/B LC gene resulted in a 50% higher yield of protein expression and/or recovery than previously reported [32]. We produced a recombinant protein in a correctly folded state that was biologically active. Primers for amplifying the nucleotide sequence encoding the BoNT/B LC were constructed on the basis of the Minton sequence [35]. Oligonucleotides were designed to contain *Nde*I–*Bam*HI restriction sites that facilitated the insertion of the 1323 nucleotide sequence in frame with the pET24a+ parent vector beginning with an ATG start codon. At the protein level the sequence shared 100% identity to the Minton sequence.

Expression and purification of the BoNT/B LC

Upon induction by the addition of IPTG at 18 °C, BoNT/B LC was over-expressed. The recombinant protein was solubilized in lysis buffer before sonication to reduce the amount of cell debris and nucleic acid. A clear lysate was obtained after centrifugation which represents the crude protein solution and contains the BoNT/B LC in the soluble fraction. The soluble protein was purified to near homogeneity by two rounds of cation exchange chromatography using a Poros HS 20 followed by a third pass through cation exchange chromatography using a Source S column (Figs. 1A and B); peak fractions were collected and analyzed for purity. Verification of the purified 50 kDa protein was done by SDS–PAGE using Coomassie brilliant blue R-250 stain (Fig. 2A) and SilverXpress silver stain (Fig. 2B) and Western blot analysis using a horse polyclonal antibody (Fig. 2C). The efficiency of the purification process is shown in Table 1. Protein obtained from a 1 L culture typically yielded $\geqslant 12$ mg which was greater than 98% pure based on densitometry analysis of the silver stained gel.

N-terminal sequence and mass spectroscopic analysis of rBoNT/B LC

The molecular mass of the purified recombinant BoNT/B LC was determined by surface enhanced laser desorption/ionization (SELDI)-TOF analysis. It was 50.8 kDa which corresponded to the predicted molecular mass based on the amino acid sequence. Matrix assisted laser desorption/ionization (MALDI)-TOF was also preformed and 85% of the amino acid sequence was observed. To verify whether the LC contains the initiating methionine residue, N-terminal sequencing analysis was performed on the first 10 residues of the protein. The N-terminal amino sequence of recombinant BoNT/B LC was PVTINNFNYN as expected. We observed that the initiating methionine residue of the protein had been removed by *E. coli* methionyl aminopeptidase.

Isoelectric focusing

The isoelectric focusing analysis of the recombinant BoNT/B LC revealed a p*I* of 7.25 which is higher than the calculated theoretical isoelectric point of 6.3 (http://us.expasy.org). The experimental p*I* value for the native light chain was equal to the calculated p*I* 6.3. A number of methods have been proposed for the theoretical determination of the p*I*s of proteins [36,37]. Typically, these methods give results that are within ± 1 pH unit of the experimental p*I*. When calculating theoretical p*I* values, calculated p*I*s often disagree with experimentally measured p*I*s [38]. The theoretical p*I* estimate of 6.3 assumes that all residues have pK_a values that are equivalent to the isolated residues. The actual p*I* value for a protein is affected by the tertiary structure of the protein, which can lead to differences between calculated and experimental p*I* values. A change in the p*I* value (6.3–7.25) may be the result of masking of carboxyl groups in the tertiary structure of the protein [39].

Structural features of LC at different pH values and in the presence of zinc

To detect and examine any conformational changes induced by pH differences, buffer and zinc, we employed far-UV circular dichroism and tryptophan fluorescence emission spectroscopies in the absence and presence of 0.5 mM $ZnCl_2$ in 46 mM Pipes, pH 6.5, and in the absence of $ZnCl_2$ in 46 mM Mes, pH 5.5 or 46 mM Hepes pH 8.0 (Fig. 3). The CD spectra were similar in the four conditions displaying the characteristic double minima at 208 and 220 nm as expected (Fig. 3A). This indicates that the secondary structure of BoNT/B LC was similar in all conditions. The tryptophan fluorescence emission spectra were similar in all conditions tested. However, the addition of 0.5 mM $ZnCl_2$ increased the fluorescence intensity about 11% as compared to the fluorescence intensity at pH 6.5 without affecting the emission maximum or shape of the spectrum (Fig. 3B). The fluorescence intensity decreased

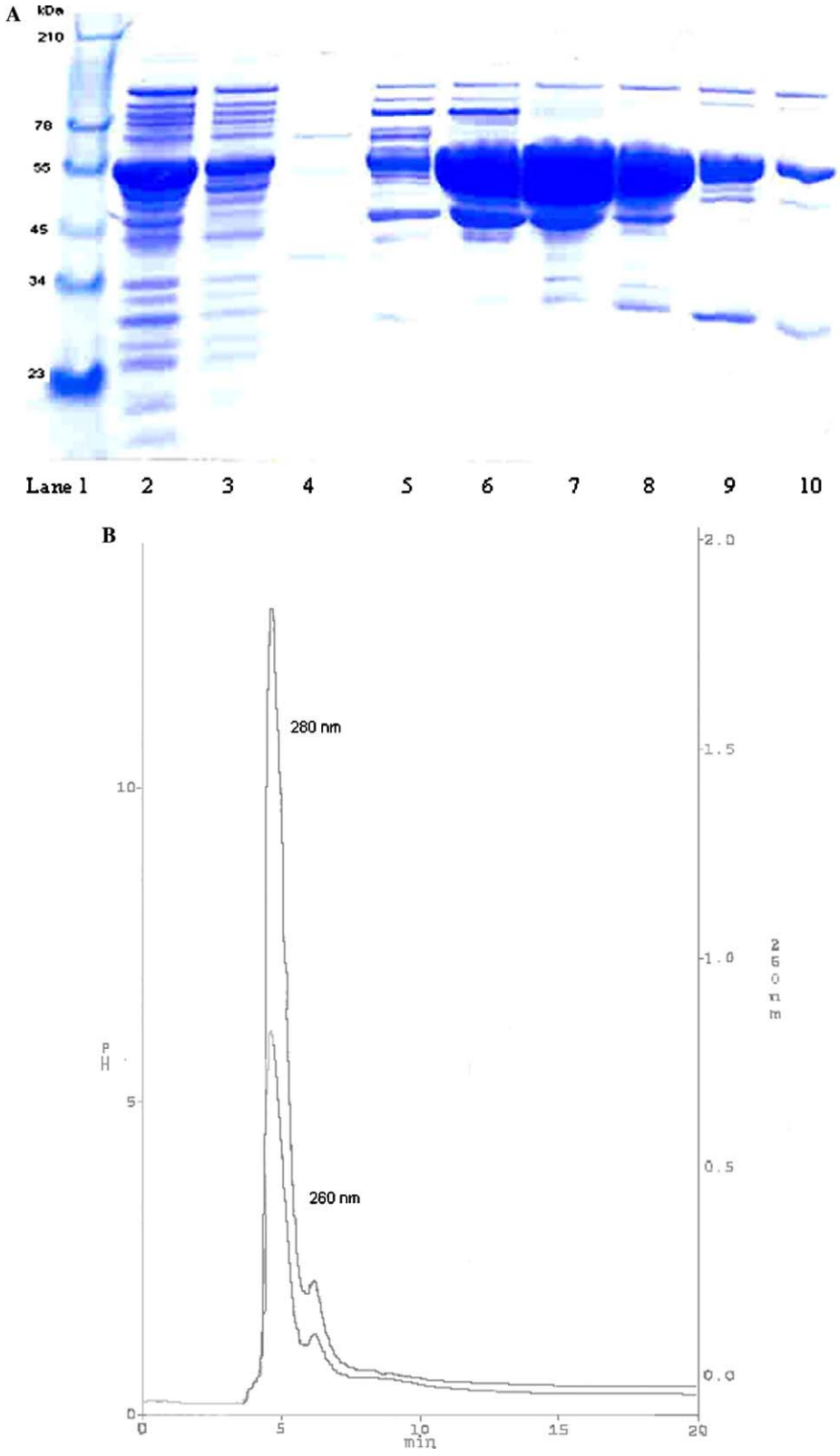

Fig. 1. (A) First round purification of BoNT/B LC from *E. coli*. Recombinant BoNT/B LC was purified from *E. coli* cell paste using a three column strategy described in Results. Protein samples were separated by SDS–PAGE and visualized by Coomassie stain. Invitrogen's pre-stained SeeBlue molecular weight markers were used (15 μl lane 1). Clarified crude lysate (lane 2) was loaded onto Poros HS 20 column. Lane 3 represents the flow through, lanes 4 and 5 represent fraction 21 and 22, respectively. Lanes 6, 7, and 8 represent fractions 23, 24, and 25 respectively which were pooled and subjected to another round of cation exchange chromatography. Lane 9 represents fraction 26 and lane 10 represents fraction 27. (B) Purification chromatogram for the recombinant protein LC column Poros HS 20.

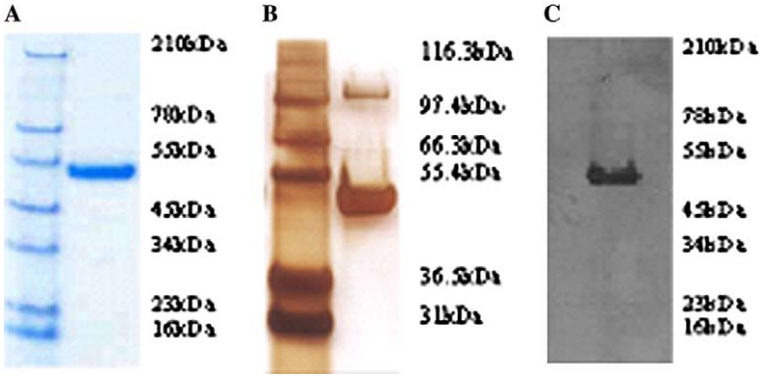

Fig. 2. (A) Reducing SDS–PAGE of purified BoNT/B LC separated on a 10% tricine gel and visualized by Coomassie blue stain; Invitrogen's pre-stained SeeBlue molecular weight markers were used (15 μl/lane 1). (B) Silver stain analysis of purified BoNT/B LC following SDS–PAGE. (C) Western blot analysis of purified BoNT/B LC detected with polyclonal horse BoNT/B antibody.

Table 1
Purification of recombinant BoNT/B LC from the soluble fraction

Steps	Total protein (mg)[a]	Step yield (%)	Overall yield (%)
Soluble fraction	91	100	100
1st ion exchange HS 20	16	17	17
2nd ion exchange HS 20	10	80	9
3rd ion exchange sources 15 S	4	95	4

Data are normalized to quantities obtained per gram of cell paste.
[a] Total protein was determined by BCA assay (Pierce) with BSA as a standard.

about 11% as compared to the fluorescence intensity at pH 6.5 without affecting the emission maximum or shape of the spectrum when the protein was at high pH in the absence of $ZnCl_2$ (Fig. 3B). The blue-shifted tryptophan fluorescence emission spectra with a λ_{max} of 318 nm (λ_{max} of free tryptophan is about 354 nm) suggests that the single tryptophan residue (W44) is buried in a hydrophobic environment that is not significantly affected by either pH or zinc. The results of the CD and fluorescence experiments suggest that pH changes over the range of 6.5–8.0 and the addition of 0.5 mM $ZnCl_2$ have no significant effect on secondary and tertiary structures of BoNT/B LC.

We can also conclude from the data that the LC remains stable up to 40 °C with pH values in the range of 6.5–8.0. Estimation of the secondary structure at 20 °C represents the native structure of the LC. Alterations in the secondary structure content of BoNT/B LC at 20 °C with respect to various conditions are listed in Table 2. When the pH rises there is a marginal increase in the α-helix content of the LC and a marginal decrease in the β-sheet content is observed. The addition of zinc also appears to increase the α-helix content of the LC as well as decrease the β-sheet content. There is no significant change in the content of turns or random coils of the LC under all conditions tested.

Properties of the zinc-metalloprotease activity of the BoNT/B LC

In order to determine the optimum conditions for the metalloprotease activity of BoNT/B LC, several parameters were examined (Fig. 4). The pH dependence on the cleavage of 35-residue peptide of human VAMP-2 (HV35) by BoNT/B LC was assessed using three buffers, Mes, Pipes, and Hepes (Fig. 4A). Optimal activity of the protease activity of BoNT/B LC was found at near-neutral pH with a maximum between 6.6 and 7.3 (Fig. 4A). The rate of cleavage of peptide HV35 was markedly reduced at pH values higher than 7.5 and lower than 6.0. Although pH variations could cause changes in the substrate diminishing the cleavability in a pH-dependent fashion, the small level of activity remaining at pH values higher than 7.5 and lower than 6.0 could be accounted for by a change in the ionization of the histidine residues which co-ordinate the zinc molecule in the catalytic mechanism of BoNT/B LC. Fig. 4A also demonstrates that the rate of cleavage of HV35 by BoNT/B LC was influenced by the type of buffers used in the reaction mixtures. Cleavage rates of the peptide in PIPES were lower than that observed with Hepes and Mes. For comparison, the optimum pH values of BoNT/A LC [40] and BoNT/B [41] are 7.0–7.5 and 6.5–7.0, respectively. Since the highest rates of metalloprotease activity by BoNT/B LC were observed in Hepes buffer, this buffer was used throughout assay experiments. The metalloprotease activity of BoNT/B LC was also influenced by temperature; being more active at 37 °C (Fig. 4B). Similar results were noted for BoNT/A LC and BoNT/B. It is uncertain whether this result is a reflection of the thermal conformational stability of BoNT/B LC or substrate HV35. The effect of ionic strength on metalloprotease activity of BoNT/B LC was also studied. Increasing the concentration of sodium chloride in 50 mM Hepes buffer had markedly inhibited the protease activity of BoNT/B LC (Fig. 4C). Similar results with respect to the effect of ionic strength have been reported for the cleavage of HV35 by BoNT/B [30].

Thermal denaturation of BoNT/B LC

To probe the pH and $ZnCl_2$ affect on LC stability, unfolding of LC was examined by monitoring the CD signal at 222 nm as a function of temperature. Fig. 5 shows the thermal transition curves of LC under different

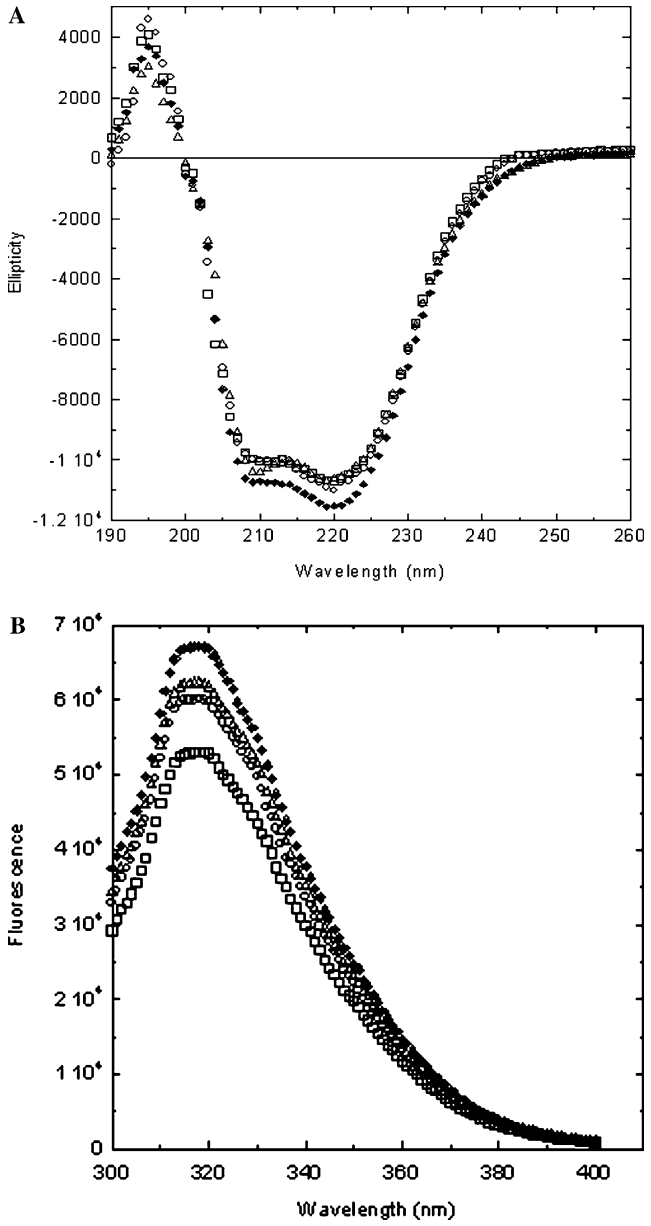

Fig. 3. Far UV circular dichroism (A) and tryptophan fluorescence emission (B) spectra of BoNT/B LC in the absence (circle) and presence of 0.5 mM $ZnCl_2$ (closed diamond) in 46 mM Pipes, pH 6.5, and in the absence of $ZnCl_2$ in 46 mM Mes, pH 5.5 (triangle) and in 46 mM Hepes, pH 8.0 (open square). Protein concentration in these experiments was 0.15–0.19 mg/ml.

Table 2
Calculated secondary structural content of BoNT/B LC under various conditions

Buffer	pH	Addition	% α-helix	% β-sheet	% turns	% random coil
Mes	5.5	None	16.5	31	28.5	24
Pipes	6.5	$ZnCl_2$	20	26.5	29	24.5
Pipes	6.5	None	18.5	28	29.5	24
Hepes	8.0	None	18	29	29	24

Secondary structural contents were calculated by SELCON using the Softsec program (Softwood).

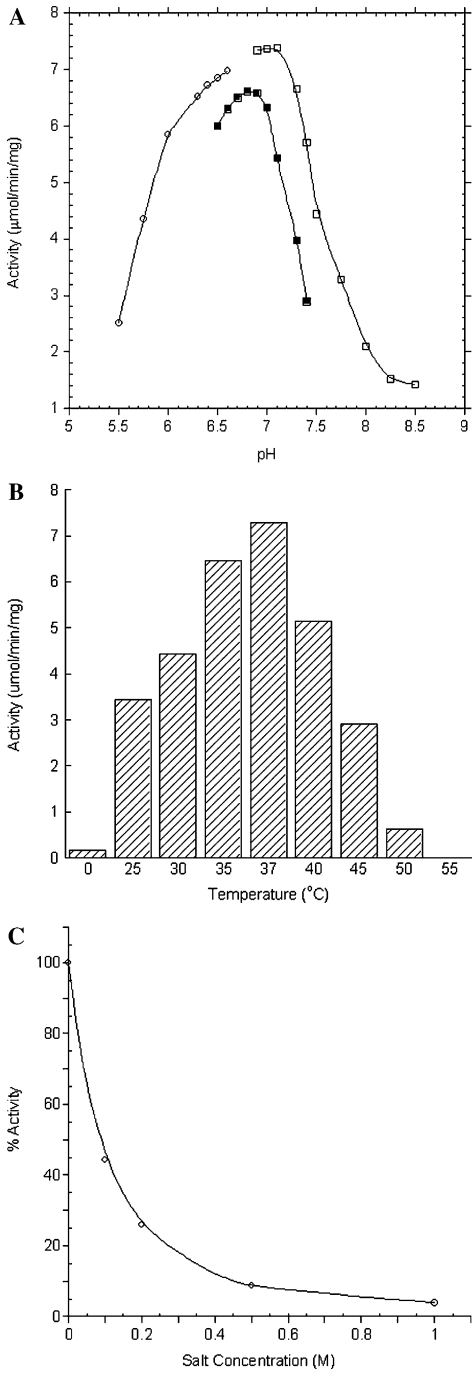

Fig. 4. Effect of pH, temperature, and ionic strength on the metalloprotease activity of BoNT/B LC. (A) The pH dependence on the cleavage of the synthetic, VAMP-derived substrate (HV35) by BoNT/B LC was assessed using three buffers, Mes (circle), Pipes (closed square), and Hepes (open square). Assays contained 0.2 mM peptide substrate, 1.0 μg LC and 50 mM buffer at various pH values. Each data point represents an average of three determinations. (B) Reaction mixtures (0.03 ml) containing 50 mM Hepes pH 7.2, 0.18 mM substrate, and 1.0 μg BoNT/B LC incubated at the indicated temperature for 5 min. Each point was performed in triplicate and the maximum activity (100%) was 7.4 μmol/min/mg LC at 37 °C. (C) Reaction mixtures (0.03 ml) containing 50 mM Hepes, pH 7.2, 0.18 mM substrate, 1.0 μg BoNT/B LC, and the appropriate NaCl concentrations were incubated at 37 °C for 5 min. Each point was performed in triplicate and the maximum activity (100%) was 7.3 μmol/min/mg LC in the absence of NaCl.

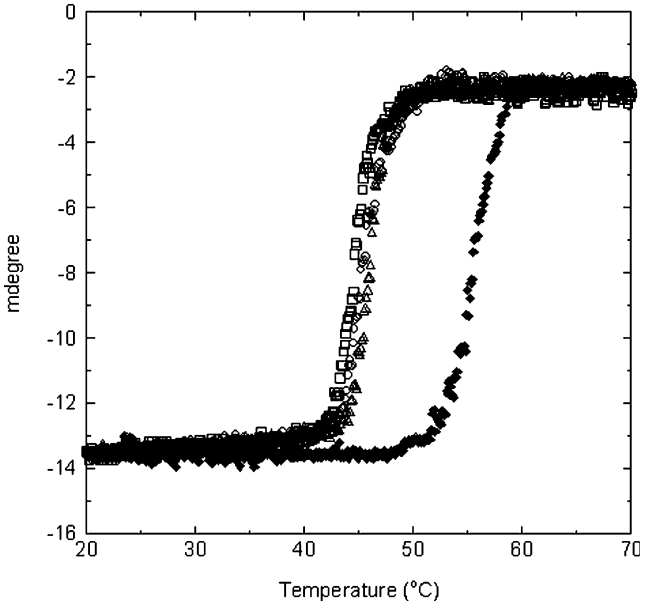

Fig. 5. Thermal unfolding of BoNT/ B LC in the absence (circle) and presence of 0.5 mM $ZnCl_2$ (closed diamond) in 46 mM Pipes pH 6.5 and in the absence of $ZnCl_2$ in 46 mM Mes pH5.5 (triangle) and in 46 mM Hepes (open square) at 222 nm. Protein concentration in these experiments was 0.15–0.19 mg/ml. T_m (the midpoint of thermal transition) values were calculated as 46 °C at pH 5.5, 6.5, and 8.0 in the absence and 56 °C at pH 6.5 in the presence of 0.5 mM $ZnCl_2$.

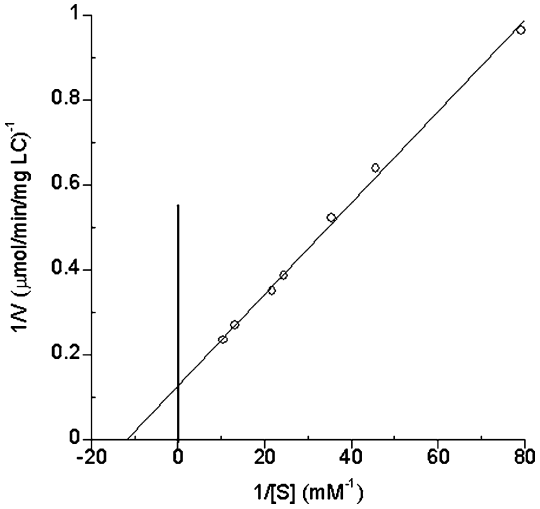

Fig. 6. Determination of K_m and V_{max} from the double-reciprocal (Lineweaver–Burke) plot of initial rates of proteolysis versus seven different substrate concentrations ranging from 0.016 to 0.1 mM by BoNT/B LC. Assay mixtures (30 μl) contained 50 mM Hepes buffer (pH 7.2) and 1.0 μg/ml BoNT/B LC. The K_m and V_{max} were calculated as 0.083 mM and 7.8 μmol /min/mg LC, respectively.

pH conditions and in the presence of 0.5 mM $ZnCl_2$. Although the secondary structure was not affected by zinc and different pH conditions, the unfolding pattern of LC determined in the presence of $ZnCl_2$ differed significantly. Sharp and monophasic denaturation curves were obtained in the absence and presence of 0.5 mM $ZnCl_2$ in 46 mM Pipes, pH 6.5, and in the absence of $ZnCl_2$ in 46 mM Mes, pH 5.5, or 46 mM Hepes, pH 8.0, indicating that the LC preparation was homogeneous in all conditions. The thermal denaturation curves allowed calculation of the apparent melting temperature T_m (midpoint of thermal transition) values. The denaturation curves of the LC in the absence of $ZnCl_2$ at pH 5.5, 6.5, and 8.0 coincided and yielded a T_m of 46 °C. LC at pH 5.5, 6.5, and 8.0 in the presence of $ZnCl_2$ yielded a T_m of 56 °C. We can conclude from this data that the presence of $ZnCl_2$ appears to significantly stabilize the LC as indicated by an increase in T_m by 10 °C.

Enzyme kinetics of BoNT/B LC

To determine the K_m and V_{max} values initial rates of proteolysis were determined in triplicate for various concentrations of the substrate (0.016–0.1 mM) and results were plotted as $1/V$ versus $1/S$ (Fig. 6, Lineweaver–Burke plot). Under the conditions of the assays, the recombinant light chain cleaved the substrate with a K_m of 0.083 mM, a V_{max} of 7.8 μmol/min /mg LC, and the K_{cat} was determined to be 40/s. For comparison, the K_m of the native dichain toxin is reported to be 0.33 mM ($k_{cat} = 24$/s) [30]. The lower K_m for the LC may be due to a more exposed active site in the free LC than in the LC of the native toxin where the active site is surrounded by the translocation domain belt and the long axis of the translocation domain [42]. Thus, the catalytic efficiency k_{cat}/K_m of the LC, 482 $[(M^{-1}s^{-1}) \times 10^3]$ which is consistent with the reported value [43], is higher than that of the native toxin, 72 $[(M^{-1}s^{-1}) \times 10^3]$ [30].

Zinc-enhanced fragmentation experiments

To test the stability of the protein, as well as examine the protein for fragmentation similar to that of BoNT/A LC as reported previously [40], protein was stored in aliquots and left at 23 or 4 °C for a predetermined time. SDS–PAGE load buffer was added to an aliquot before running the protein on a 10% tricine gel. The protein stored at 4 °C was stable for a period of 110 days in the presence or absence of $ZnCl_2$ and no fragmentation was observed (not shown). However, the protein stored at 23 °C demonstrated truncation of the LC when the protein was incubated in the presence of $ZnCl_2$ (Fig. 7) at day 25. Examination of BoNT/B LC stored with $ZnCl_2$ at room temperature for a shorter period of time indicated that fragmentation can be detected. Similar results have been reported for BoNT/A LC [26].

We also tested the activity of the protein at the end point and found that the protein stored in 50 mM sodium phosphate, pH 6.5 at 4 °C retained 100% of its initial catalytic activity while the protein stored at 23 °C, in the same buffer, retained 90% of its enzymatic activity.

Immunogenicity studies

Animal protection studies were done to determine if the purified recombinant BoNT/B LC has the ability to elicit

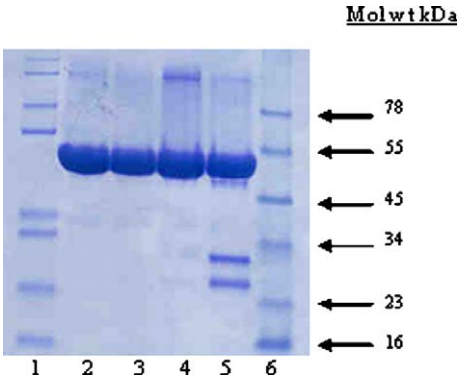

Fig. 7. BoNT/B LC in 50 mM Na-phosphate buffer pH 6.5 in the presence and absence of 0.5 mM $ZnCl_2$ was aliquoted and left at 23 °C temperature for 25 days. Lane 1, Invitrogen's Mark12 molecular weight marker (15 μl); lane 2, day zero, BoNT/B LC in 50 mM Na-phosphate buffer, pH 6.5, in the absence of $ZnCl_2$; lane 3, day zero, BoNT/B LC in 50 mM Na-phosphate buffer, pH 6.5, in the presence of $ZnCl_2$; lane 4, day 25, BoNT/B LC in 50 mM Na-phosphate buffer, pH 6.5, in the absence of $ZnCl_2$; lane 5, day 25, BoNT/B LC in 50 mM Na-phosphate buffer, pH 6.5, in the presence of $ZnCl_2$; lane 6, Invitrogen's SeeBlue molecular weight marker (15 μl); SDS–PAGE load buffer was added to the sample before running on a 10% tricine gel. Protein stored at 23 °C demonstrated truncation of the LC when the protein was incubated in the presence of $ZnCl_2$ (lane 5) at day 25.

protective immunity in mice. Three groups of 20 mice were used for each protection assay. Two groups of 20 mice were injected with the LC i.m.; one group with 5 μg/mouse and one group with 15 μg/mouse. Each group received three injections, were bled, and subsequently challenged with BoNT B toxin by i.p. administration (Table 3). Within each group of 20, 10 mice were challenged with 1×10^2 and 10 mice were challenged with 1×10^3 MLD50 holotoxin. Survivor data is recorded as the number of survivors from the total number of animals tested. Naïve mice were challenged with the same levels of toxin and used as control. Mice were observed daily and the number of mice that survived after 5 days was recorded. None of the control animals survived when toxin was administered. Mice that received three injections of 5 μg of purified soluble BoNT/B LC were completely protected when challenged with i.p. 10^2 or 10^3 LD_{50} of BoNT/B toxin. Three doses of 15 μg of purified BoNT/B LC protected mice from a challenge of 10^2 or 10^3 LD_{50} of

Table 3
Survival of challenged mice vaccinated with BoNT/B LC from *E. coli*

Mice	Challenged with 10^2 MLD_{50} BoNT/B survivors/challenged	Challenged with 10^3 MLD_{50} BoNT/B survivors/challenged
Group $I_{(5\mu g)}$	10/10	10/10
Group $II_{(15\mu g)}$	9/10	10/10
Group $III_{(naive)}$	0/10	0/10

Mice in group I received 5 μg of purified recombinant BoNT/B LC adsorbed to Alhydrogel, injections were at 0, 2, and 6 weeks. Group II received 15 μg of purified recombinant BoNT/B LC adsorbed to Alhydrogel, injections were at 0, 2, and 6 weeks. Group III were naïve mice. Mice were immunized and challenged by administration of neurotoxin. Mice were observed daily and the number of mice that survived after 5 days was recorded.

Table 4
Correlation of individual ELISA titers with survival after vaccination with purified recombinant BoNT/B LC

Individual ELISA titer[a]	Survival (no. alive/total)[b]	% survival
⩽100	27/28	96
400	25/25	100
⩾1600	7/7	100

[a] Serum was bled from each mouse individually. Titer is the reciprocal of the highest dilution having an A_{405} of greater than 0.2 after correction for background.

[b] Mice were challenged with 10^2 or 10^3 i.p. LD_{50} of BoNT/B 7 days after the last vaccination.

native BoNT/B toxin with the exception of one mouse (Table 4). Individual serum antibody ELISA titers of mice injected with soluble BoNT/B correlated with survival as all mice with ELISA titers of 100 or greater survived toxin challenge (Table 4). Mice with titers less than 100 did not survive. The work presented here demonstrates that purified BoNT/B LC has the capacity to protect against a challenge dose of neurotoxin.

Conclusions

This paper describes the expression of BoNT/B LC in *E. coli* using an optimized synthetic gene to produce a highly pure, 50 kDa protein. The growth and induction conditions for expression were optimized to obtain this protein in the soluble fraction. Traditional ion exchange chromatography methods were used to purify a protein that is highly active. CD analysis and tryptophan fluorescence emission spectroscopy indicate that the LC possesses a defined set of secondary and tertiary structures. The only Trp residue is in a hydrophobic environment as indicated by a blue-shifted emission λ_{max} of 318 nm. The CD analysis indicates that the secondary structure of the light chain consists predominantly of β-sheets which is consistent with the known structure of the recombinant BoNT B heavy chain [44]. We also conclude that the Trp residue is constrained in the protein hydrophobic core, a feature supported by the published X-ray crystallography structure of BoNT/B [42,45]. The unfolding of BoNT/B LC at different pH values demonstrate that pH has no significant effect, however, the addition of zinc increases the thermal stability of the LC at pH 6.5. We have also demonstrated that 50 mM Hepes, pH of 7.2–7.3 is the best system among the buffer systems tested to obtain a maximum enzymatic activity. Kinetic analysis demonstrated that the catalytic efficiency of the LC is higher than that of the native dichain toxin. This may indicate that the LC has a more exposed active site in free LC than in the LC of the native toxin where the active site is surrounded by the translocation domain belt and the long axis of the translocation domain [42]. The purified BoNT/B LC was stable for at least 110 days when stored at 4 °C in 50 mM sodium phosphate, pH 6.5, remained fully soluble, and retained its initial catalytic activity.

Like BoNT/A LC, BoNT/B LC expressed and purified from *E. coli* is nontoxic when injected into mice at a concentration of 5–15 μg of LC per mouse [40]. Table 4 shows that all mouse antisera tested had titers against BoNT/B. With the exception of one mouse, which had a titer below 100, the mice were protected against subsequent challenge with low doses of BoNT/B toxin. In contrast, purified BoNT/A LC failed to protect even when titers were boosted with adjuvant [40].

In conclusion we have expressed and purified $\geqslant 4$ mg/g quantities of recombinant BoNT/B LC and characterized the protein. The protein is soluble, nontoxic, catalytically active and highly stable making it ideal for investigators to identify potential inhibitors.

Acknowledgments

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